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부분 방광출구폐색 흰쥐 모델에서 폐색 해소 후 산소유리기제거제가 방광의 형태학적 및 기능적 변화에 미치는 예방적 효과

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지도교수 오승준

이 논문을 추민수박사 학위논문으로 제출함

2018년 10월

서울대학교 대학원

의학과 비뇨의학 전공

추 민 수

추민수의 박사 학위논문을 인준함

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Preventive Effect of an Oxygen Free Radical Scavenger on Morphological and Functional Changes after Relief of Partial Bladder Outlet Obstruction in Rat Urinary Bladder

by
Min Soo Choo, MD

A Thesis Submitted to the Department of Urology in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Medicine (Urology) at the Seoul National University College of Medicine

January 2019

Approved by thesis committee:

Professor _____ Chairman

Professor _____ Vice Chairman

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Professor _____

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Abstract

Preventive Effect of an Oxygen Free Radical Scavenger on Morphological and Functional changes after Relief of Partial Bladder Outlet Obstruction in Rat Urinary Bladder

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Department of Urology,

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To investigate the effect of a free radical scavenger (tempol) after relief of partial bladder outlet obstruction (pBOO) on bladder function in a rat model.

pBOO was induced in 50 eight-week-old female Sprague-Dawley rats and relieved three weeks later. The rats were divided randomly into five groups: sham-operated, tempol-treated for one week (Treat-1w) or three weeks (Treat-3w), and no treatment for one week (unTreat-1w) or three weeks (unTreat-3w). Awake cystometries were obtained one

or three weeks after relief according to the grouping. The bladders were isolated and weighed. Oxidative stress was assessed using malondialdehyde. Hematoxylin and eosin, Masson's trichrome and TUNEL staining were used to analyze histological changes. Expression of beta-3 adrenoreceptor was examined by Western blotting.

The tempol-treated groups showed significant decreases in the malondialdehyde concentrations at both one and three weeks after relief of pBOO. The thickness and collagen fiber deposition of the detrusor muscle layer were significantly decreased in the treated groups. The tempol-treated groups exhibited a significant decrease in the number of non-voiding contractions per voiding cycle (unTreat-1w vs. Treat-1w, 1.2 ± 0.8 vs. 0.4 ± 0.4, P = 0.010; unTreat-3w vs. Treat-3w, 1.5 ± 0.7 vs. 0.2 ± 0.3, P = 0.002). Apoptosis was mainly observed in the urothelial cell layer, and the rate of apoptosis was significantly decreased in the treated groups (48.9 ± 3.4% vs. 32.7 ± 11.1%, P = 0.024; 25.8 ± 4.7% vs. 15.7 ± 9.8%, P = 0.314). Expression of beta-3 adrenoreceptor was increased in tempol-treated rats.

Ischemia-reperfusion injury after relief of pBOO caused histological and functional changes in the bladder. Free radical scavenger treatment prevented this oxidative stress.

*A significant part of this work was published in PLoS One journal (2018;13(10):e0199800).

Keywords: urinary bladder neck obstruction; prostate hyperplasia; urinary bladder, overactive; oxidative stress; free radical scavengers; receptors, adrenergic, beta-3

Student ID: 2013-30575

Contents

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Abbreviations and Acronyms

BPH, benign prostatic hyperplasia

BOO, bladder outlet obstruction

CMG, cystometrogram

H&E, hematoxylin and eosin

HoLEP, holmium laser enucleation of the prostate

I/R injury, ischemia-reperfusion injury

MDA, malondialdehyde

NVC, non-voiding contraction

pBOO, partial bladder outlet obstruction

Treat-1w, tempol-treated for one week

Treat-3w, tempol-treated for three weeks

TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling

unTreat-1w, no treatment for one week

unTreat-3w, no treatment for three week

Introduction

1. Clinical significance of bladder outlet obstruction

Bladder outlet obstruction (BOO) occurs due to a variety of causes, including the posterior urothelial valve in children, urethral stricture in adults, and benign prostatic hyperplasia (BPH) in the elderly (Keihani et al.2015). Although the most common cause in the clinic is BPH in older men, BOO may also occur in women with several anatomical and/or functional etiologies, including pelvic organ prolapse, Skene's gland cyst, primary bladder neck obstruction, and detrusor external sphincter dyssynergia (Lin et al. 2016). BOO is one of the most important clinical problems and can cause overactive bladder, urinary incontinence, urinary tract infection, vesicoureteral reflux, hydronephrosis, and renal insufficiency through chronic urinary retention (Solomon et al. 2018).

2. Mechanism of bladder changes associated with BOO

BOO causes bladder degeneration, which is known to progress through three sequential stages: hypertrophy, compensation and decompensation (Levin et al. 2004). In the first stage, the hypertrophy stage, hypertrophy of the bladder wall occurs in response to the acute distention of the bladder. The second stage is the compensation phase,

in which the enlarged bladder is functionally stable, but collagen fibers are continuously deposited on the bladder wall, leading to sustained ischemia–reperfusion (I/R) injury. Accumulation of I/R injury leads to the decompensation stage. In this final step, functional changes occur by denervation and loss of smooth muscle. Denervation due to cumulative damage can occur in both the urothelium and smooth muscle. The neuronal theory states that bladder hypersensitivity is causes by sensory nerve damage due to urothelial apoptosis (Figure 1). When overactivity occurs after sensory nerve damage, a change in the expression of the beta receptor in the urothelium is thought to occur, and this change in receptor activity may be clinically related to the drug response of beta–3 agonists.

3. Detrusor overactivity after relief of obstruction

In patients with severe degenerative bladder due to BOO, surgical treatment may temporarily exacerbate bladder storage dysfunction related to detrusor overactivity and even cause transient urgency and urgency urinary incontinence (Dybowski et al. 2014). Newly developed urgency urinary incontinence after prostatectomy procedures for BPH has been reported in 7.1 – 44.0% of patients (Cho et al. 2011). This de novo urgency urinary incontinence causes significant stress and anxiety not only for the patients but also for the surgeons because it differs from

the post-operative complication of stress urinary incontinence. In a recent prospective study of persistent storage symptoms after successful relief of BOO, urodynamic detrusor overactivity was persistent in approximately 40% of the patients (Antunes et al. 2015).

4. Cell apoptosis due to ischemia-reperfusion injury

I/R injury has been suggested to be a cause of post-operative bladder dysfunction in BPH patients (Chuang et al. 2018). In patients with chronic BOO due to BPH, a chronic ischemic status is induced in the inner bladder wall due to persistent high intravesical pressure (Gotoh et al. 2018). In this condition, relief of chronic obstruction causes reperfusion and reoxygenation, resulting in the generation of reactive oxygen species, which cause more severe oxidative damage and cell apoptosis in the bladder wall (Li et al. 2010)(Figure 2).

5. Prevention of ischemia-reperfusion injury

Efforts to prevent I/R injuries have been made in various medical fields. Several antioxidants have been used to reduce I/R injury in patients with ischemic diseases, such as coronary artery disease or stroke, and in the transplantation field. For instance, L-alanyl-glutamine attenuates I/R injury in liver transplantation patients (Barros et al. 2015). Some

antioxidants, such as CoQ10, beta carotene, lycopene, quercetin, resveratrol, vitamin C and vitamin E, have shown preventive and therapeutic benefits for different forms of cardiovascular disease (Jain et al. 2015). However, no studies have investigated methods to prevent I/R injury after BPH surgery. The effects of I/R injury after BPH surgery have only recently drawn researchers' attention.

6. Purpose of this research

Our research team revealed in previous studies that apoptosis occurs due to repeated I/R injury during the process of bladder retention and subsequent emptying, and that apoptosis pathways such as PARP or JNK are involved in this process (Li et al. 2010; Li et al. 2010; Li et al. 2011). Based on these findings, a hypothesis that administration of a free radical scavenger can prevent I/R injuries which occur after relief of partial BOO (pBOO) has been proposed. The aim of this study was to evaluate the preventive effect of an oxygen free radical scavenger, tempol, on morphological and functional bladder changes in oxidative stress after relief of pBOO in a rat model.

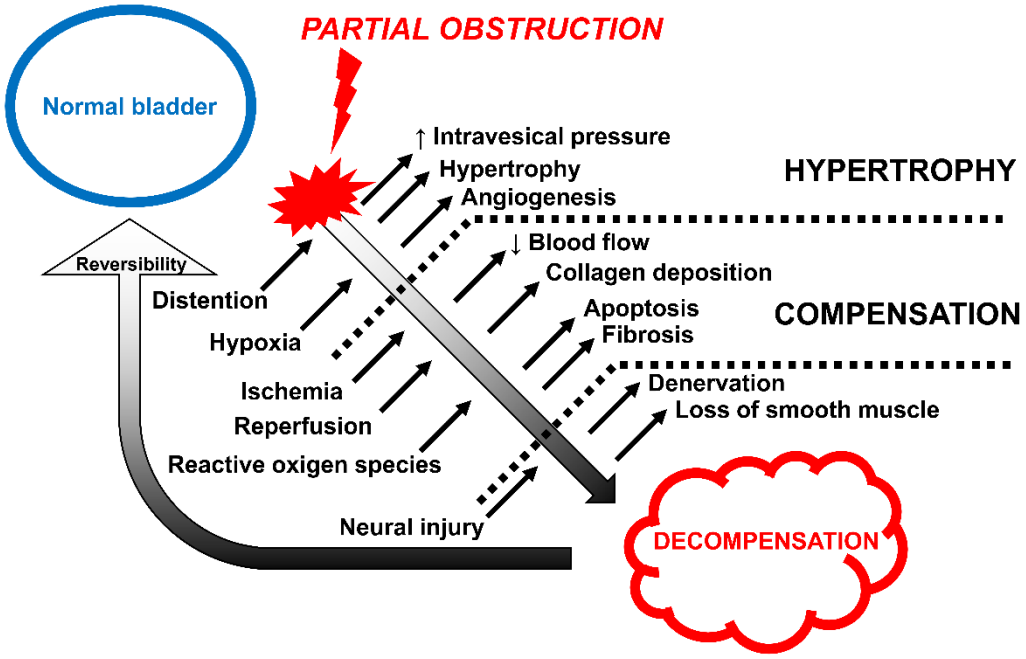


Figure 1. A flow diagram demonstrating the sequential stages of progression of bladder degeneration in response to partial bladder outlet obstruction and the major changes in each stage (Zderic et al. 2012).

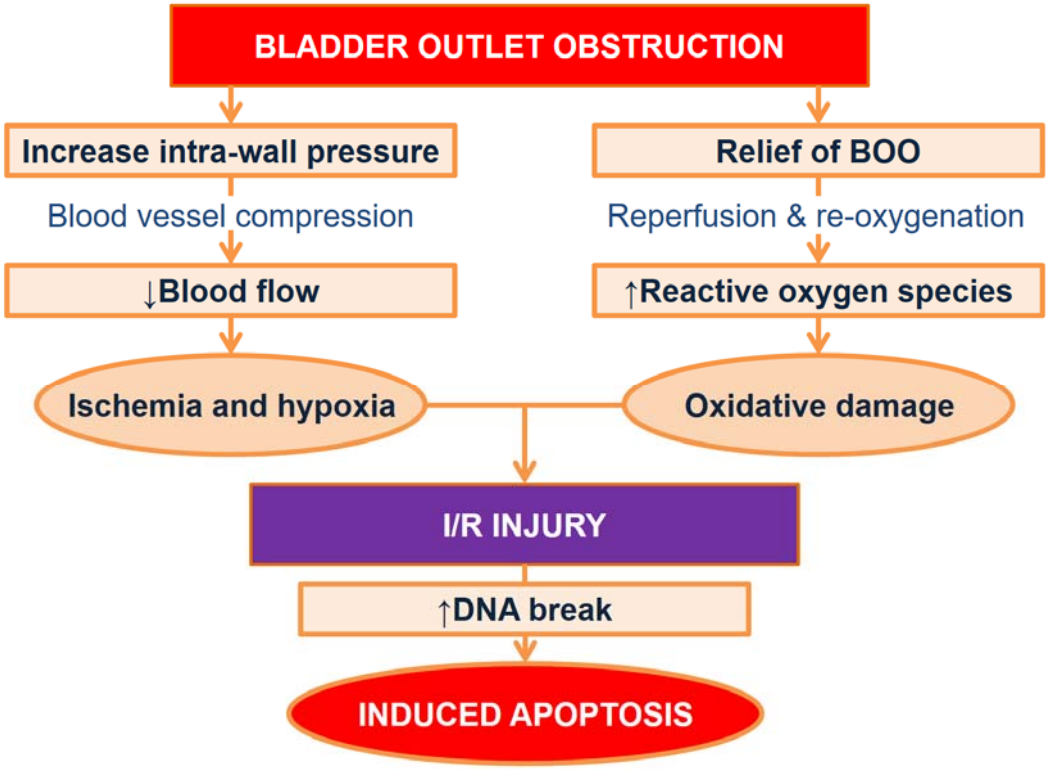


Figure 1. Biochemical mechanisms of bladder damage caused by partial bladder outlet obstruction and its relief. BOO, bladder outlet obstruction; I/R injury, ischemia–reperfusion injury.

Materials and Methods

1. Animals and study design

Eight-week-old female Sprague-Dawley rats, habituated for one week and weighing between 220 and 250 g, were used in this study. Before surgery, seven-week-old rats were housed in a vivarium with free access to food and water in a light-controlled room with a diurnal cycle for one week. After surgery, the animals were caged individually and maintained under the same conditions. All animal handling and treatment procedures were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Seoul National University Hospital Institutional Animal Care and Use Committee (Protocol Number: 13-0300-C1A1), which is an AAALAC-accredited facility.

Fifty rats were randomly divided into five groups (n = 10 rats per group) (Figure 3). The first group consisted of control sham-operated rats (control group). The other four groups underwent urethral constriction to procedurally induce pBOO, followed by a reversal operation after three weeks. The second group underwent a cystometrogram (CMG) without treatment for one week after the reversal operation (unTreat-1w group). The third group underwent a CMG without treatment for three weeks after the reversal (unTreat-3w group). The fourth group

underwent a CMG with antioxidant treatment for one week after the reversal (Treat-1w group). The fifth group underwent a CMG with antioxidant treatment for three weeks after the reversal (Treat-3w group). Tempol was used as an antioxidant because it has the advantage of being soluble in water and is widely used in rat experiments. The rats in the treated group received tempol (Sigma-Aldrich, St. Louis, MO, USA) by gavage at a dose of 1.5 mmol/kg/day dissolved in water three times per day (Wilcox et al. 2008).

2. Induction of partial bladder outlet obstruction

Anesthesia was induced by ketamine/xylazine (15 mg/kg and 5 mg/kg; intramuscular injection) and maintained with isoflurane (1-3%). After shaving the skin, asepsis was attained with a 10% povidone-iodine solution while the rat was in a dorsal recumbent position. The bladder was approached through a lower midline abdominal incision. After exposing the proximal urethra, a steel rod 0.9-mm in diameter was placed around the urethra. The bladder neck was ligated using a 3-0 silk, and the steel rod was removed. The bladder was repositioned, and the abdominal wall was closed. In the sham operation group, the bladder neck was very loosely ligated to avoid inducing any obstruction. pBOO causes changes in the structure and function of the bladder. Levin et al. reported an acute response lasting for approximately two weeks (Levin

et al. 2004). In this study, the pBOO state was maintained for three weeks to induce chronic pBOO. Three weeks after inducing pBOO, each rat underwent a reversal operation that removed the ligation in the same manner. The body weight of each rat was measured before each procedure.

3. Generation of an awake cystometry model

The catheter implantation procedures were performed two days before the functional evaluation. Polyethylene catheters (PE-50; Clay-Adams, Parsippany, NJ, USA) with a cuff were inserted into the dome of the bladder through a lower abdominal incision with a purse-string suture, and the other balloon catheter simultaneously placed on the posterior side of the bladder to measure intra-abdominal pressure. The balloon and catheter were filled with distilled water, and the distal end of the catheter was sealed. Both catheters were tunneled subcutaneously to the skin of the back and anchored with a silk ligature (Figure 4a). The free end of the catheter was sealed. Each rat was housed individually after the procedures and maintained in the manner described above.

4. Functional evaluation of the voiding cycle

CMGs were performed on awakened, unanesthetized, and unrestrained

rats in metabolic cages after a minimum of two days of recovery from catheterization (Figure 4b). The bladder catheter was connected via a 3-way stopcock to a pressure transducer (Research Grade Blood Pressure Transducer; Harvard Apparatus, Holliston, MA, USA) and a microinjection pump (PHD22/2000 pump; Harvard Apparatus). Another pressure transducer was connected to an intra-abdominal balloon catheter to independently record the intra-abdominal pressure. The micturition volumes were recorded with a fluid collector connected to a force displacement transducer (Research Grade Isometric Transducer; Harvard Apparatus). Room-temperature saline was infused into the bladders at a rate of 0.4 mL/min. Pressures and micturition volumes were recorded continuously with a computerized system (PowerLab, ADInstruments, Colorado Springs, CO, USA) at a sampling rate of 50 Hz. Non-voiding contractions (NVCs) during the filling phase were defined as an increment of intravesical pressure that exceeded 2 cmH_2O from the baseline without simultaneous changes in intra-abdominal pressure and without fluid expulsion from the bladder (Kang et al. 2011).

When the bladder contractions became stable, at least five micturition cycles were recorded for each rat. The following CMG parameters were measured, and the mean value of each variable was calculated for the analysis: basal pressure (the lowest pressure during filling), threshold pressure (the pressure immediately before the initiation of micturition), peak micturition pressure (the maximum pressure during micturition),

micturition interval (the interval between micturition contractions), micturition volume, micturition duration (the time from initiation to finish of one micturition cycle), post-voided residual volume (the remaining urine after voiding), bladder capacity (the infused volume immediately before the initiation of micturition), bladder compliance (calculated by dividing the micturition volume by the difference between resting and threshold pressures), and the frequency of NVCs (per micturition cycle) (Figure 5).

5. Histological and immunohistochemical evaluation

The rats were euthanized after completion of the functional study. The whole bladder was extirpated and weighed. A portion of the bladder was divided sagittally and fixed in a 4% formaldehyde solution for histological evaluation, and the other half was snap frozen immediately in liquid nitrogen and then stored at −80°C until measurement of malondialdehyde (MDA) and the immunohistochemistry evaluation.

After obtaining 4-μm serial sections of the paraffin-embedded material, the bladder tissue sections were stained with hematoxylin and eosin or Masson's trichrome stain. The thickness of the detrusor muscle layer was evaluated in 10 randomly selected hematoxylin and eosin-stained sections. Collagen deposition was measured in 10 high-power (×400) fields from randomly selected Masson's trichrome-stained sections.

Photomicrographs were obtained using a digitalized microscopic imaging system (Nikon Eclipse 80i microscope and Nikon Digital Slight DS-U3; Nikon, Tokyo, Japan). The images were analyzed using Adobe and ImageJ software (http://rsb.info.nih.gov/ij/).

To detect apoptosis, the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method was used. In each slide, 10 high-power (×400) fields were randomly selected, and the apoptotic index was expressed as the percentage of apoptotic cells relative to the number of total cells in a given area (nonapoptotic nuclei plus apoptotic cells).

6. Measurement of malondialdehyde in the bladder

The MDA level in the bladder tissue, which is an index of oxidative stress, was determined using a commercially available kit according to the manufacturer's instructions. The MDA concentrations were normalized using the protein content (NWLSSTM Malondialdehyde Assay, Northwest Life Science Specialties, LLC, Vancouver, WA, USA).

7. Western blotting analysis of beta receptor expression

The membranes were blocked with 5% skim milk for one hour at room temperature and incubated overnight at 4°C with primary antibodies

against the beta-3 adrenergic receptors (1:1000, ab59685, Abcam), followed by incubation with appropriate horseradish peroxidase-linked secondary antibodies (1:4000) for two hours at room temperature. The bands on the blots were visualized using an enhanced chemiluminescence system (Amersham Bioscience, Buchinghamshire, UK), and densitometric analysis of the Western blots was conducted using VisionWorks LS, version 6.7.1.

8. Statistical analysis

All data are expressed as the mean and standard error of the mean (SEM). The collected data were analyzed using Student's t-test or the Mann-Whitney U test depending on whether the data followed a Gaussian distribution. A two-tailed $P < 0.05$ was considered significant. The statistical analyses were performed using IBM SPSS for Windows, Version 24.0 (IBM Inc., Armonk, NY, USA).

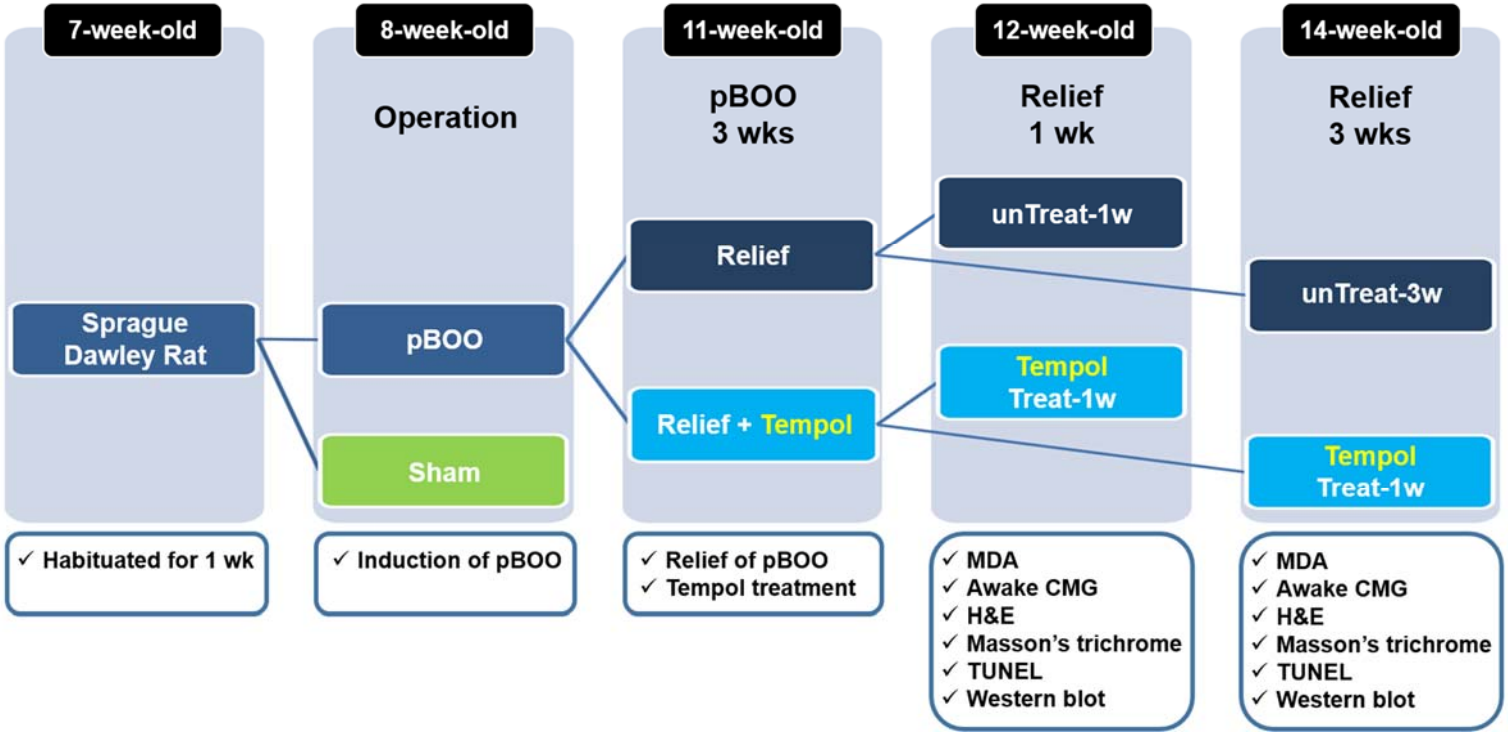


Figure 2. A schematic diagram of the experimental schedule for each group. pBOO, partial bladder outlet obstruction; MDA, malondialdehyde; CMG, cystometrogram; H&E, hematoxylin and eosin staining; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; unTreat-1w, a rat group underwent a cystometrogram without treatment for one week after the reversal operation; Treat-3w, a rat

group underwent a cystometrogram with treatment for three weeks after the reversal operation; unTreat-3w, a rat group underwent a cystometrogram without treatment for three weeks after the reversal operation.

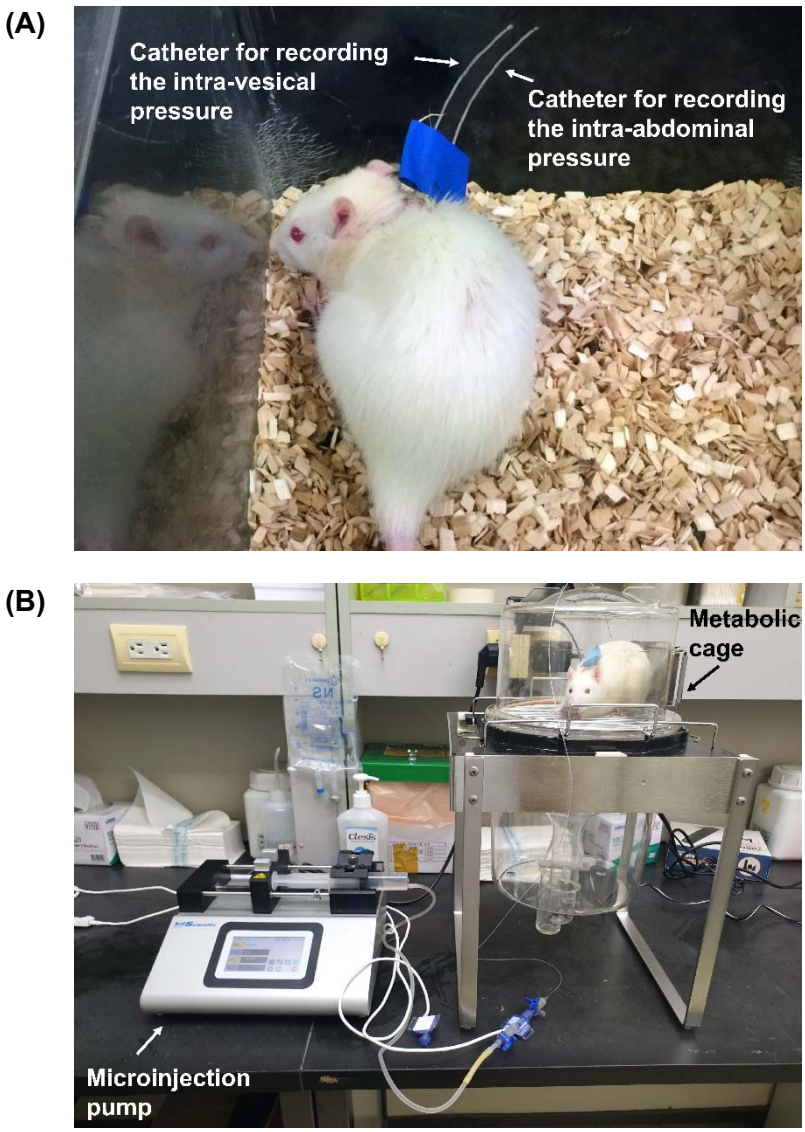


Figure 4. Awake cystometry. (A) Setup for the awake cystometry model. To simultaneously record the intra-abdominal pressure and intra-vesical pressure in rats, two catheters were placed in the back of the neck. (B) Functional evaluation of the voiding cycle. Awake cystometries were performed in a metabolic cage to simultaneously measure bladder pressure changes and urine volume in the voiding cycle while the rat was awake.

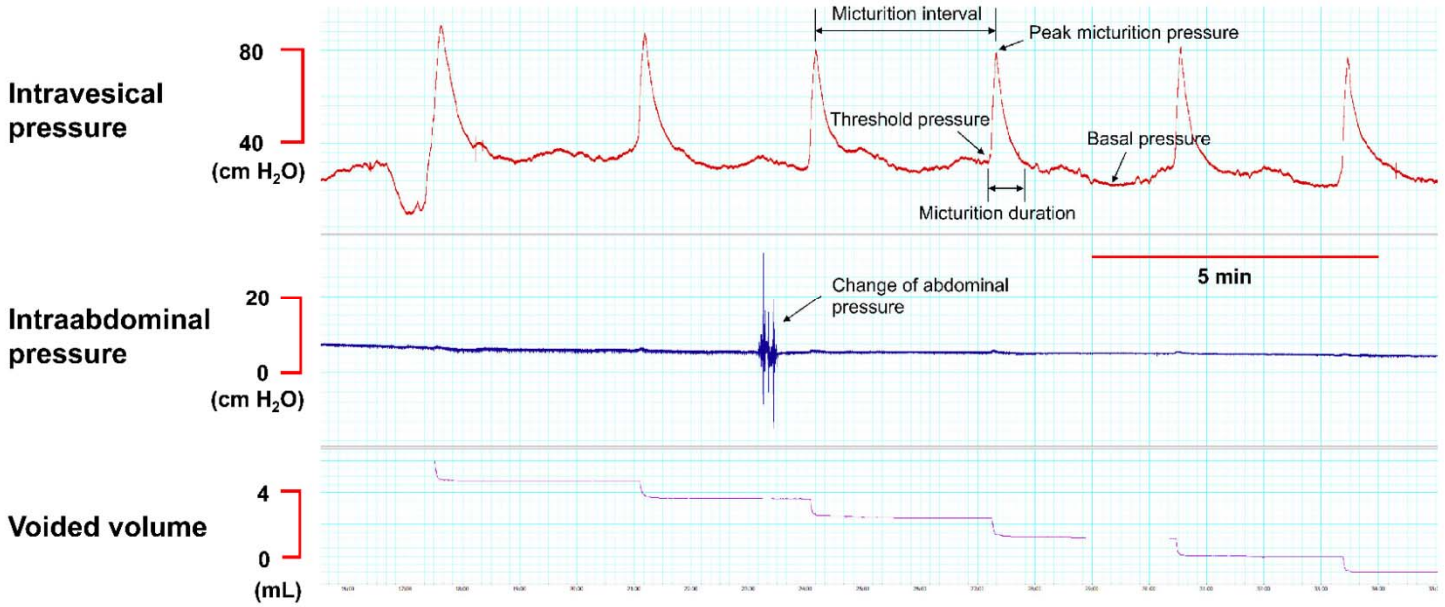


Figure 5. A representative raw trace of a normal cystometrogram. Parameters used for analysis are noted.

Results

A total of 19 rats were excluded during experiment. The results of eight rats were not used because pBOO was not properly induced. During chronic pBOO model induction, six rats died from urinary retention; three rats died due to urine leakage after tube insertion; one rat died due to a wound complication, and one rat died from infection after surgery.

1. Tempol treatment reduced ischemia-reperfusion injury in bladder tissue

MDA was measured in the bladder tissue to determine if I/R injury of the bladder was decreased by tempol treatment after relief of pBOO. The tempol-treated groups showed significant decreases in MDA concentrations at both one and three weeks after relief of pBOO compared to the concentrations in the untreated groups (unTreat-1w vs. Treat-1w, 0.76 ± 0.08 vs. 0.66 ± 0.07, P = 0.021; unTreat-3w vs. Treat-3w, 0.58 ± 0.10 vs. 0.48 ± 0.06, P = 0.030) (Figure 6). This result confirms that tempol treatment reduces I/R injury in bladder tissue at one and three weeks.

2. Morphological changes in the bladder

2.1. Ischemia-reperfusion injury increased bladder weight

No significant differences in body weight were found between the sham-operated and the pBOO-induced groups one week after relief of pBOO. However, the urinary bladder weight was significantly increased in the pBOO-induced groups compared with the weights in the sham group one week after the reversal operation (28.2 ± 8.3 g vs 12.5 ± 0.7 g, $P < 0.001$). The ratio of bladder weight to total body weight was significantly higher in the pBOO-induced groups than in the sham group.

At one week after relief of pBOO, a significant increase of the ratio was observed in the pBOO-induced groups compared to the sham group, but no difference was observed at three weeks (Table 1). The bladder weight gradually recovered to the normal range after relief of pBOO.

At both one and three weeks, bladder weight was lower in the treated group than in the untreated group, but the difference was not significant. No significant difference was found in the ratio of bladder weight to body weight between the two groups. Prevention of additional I/R injuries by tempol treatment in pBOO-denatured bladders did not result in a difference in recovery from bladder hypertrophy.

2.2. Ischemia-reperfusion injury caused detrusor hypertrophy

To evaluate the effect of I/R injury on the histological changes of the bladder, H&E and Masson's trichrome staining were performed.

Histologically, a thickened bladder wall was observed in the pBOO-induced bladder specimens. This finding was mainly due to hypertrophy of the detrusor muscle layer (Figure 7).

The thickness of the bladder wall was significantly decreased at three weeks compared to one week in both the untreated and treated groups. This result confirms that hypertrophy of the detrusor muscle is gradually resolved after the relief of pBOO.

In the comparison with the untreated group, a significant decrease was observed in the detrusor muscle thickness in the treated group after both one and three weeks of treatment (unTreat-1w vs. Treat-1w, 1164 ± 190 μm vs. 776 ± 140 μm, $P < 0.001$; unTreat-3w vs. Treat-3w, 905 ± 161 μm vs. 726 ± 162 μm, $P = 0.043$) (Figure 8). This result shows that more rapid recovery is achieved by reducing the additional I/R injury by tempol treatment after the relief of pBOO.

2.3. Ischemia-reperfusion injury caused deposition of collagen fibers in the detrusor muscle layer

In the Masson's trichrome-stained sections, tempol treatment reduced the deposition of collagen fibers in the lamina propria and detrusor muscle layer compared with the analysis in the untreated rats (Figure 9).

The ratio of collagen to smooth muscle was significantly decreased in the treated rats at one week after relief of pBOO, but the difference was not significant at three weeks (Figure 10).

3. Functional changes in the bladder

These histological findings suggest that tempol treatment can reduce I/R injury, thereby reducing additional oxidative stress and facilitating a rapid recovery from bladder degeneration.

An awake CMG was conducted to evaluate functional changes in the bladder due to I/R injury and their prevention. (Figure 11) Significant differences were found in most cystometric parameters, including the threshold pressure, micturition interval, voided volume, NVC, and bladder capacity, between the sham and the pBOO-induced groups.

A significant difference was found in the NVC incidence between the tempol-treated and the untreated groups. The number of NVCs per voiding cycle was significantly decreased in the tempol-treated groups

compared to the numbers in both the one- and three-weeks untreated groups (unTreat-1w vs. Treat-1w, 1.18 ± 0.82 vs. 0.36 ± 0.40, P = 0.010; unTreat-3w vs. Treat-3w, 1.51 ± 0.69 vs. 0.23 ± 0.25, P = 0.002). However, no other differences in the cystometric parameters were associated with tempol treatment (Table 2).

4. Mechanisms of detrusor overactivity

4.1. Tempol treatment reduced apoptosis in the urothelium

To determine pathophysiology of functional changes of detrusor overactivity, TUNEL staining was performed to confirm apoptosis.

TUNEL-positive cells were observed mainly in the urothelium but not in detrusor muscle layer (Figure 12). The numbers of TUNEL-positive cells were significantly decreased in the tempol-treated groups. This preventive effect was observed at both one and three weeks after relief (unTreat-1w vs. Treat-1w, 48.9 ± 3.4% vs. 32.7 ± 11.1%, P = 0.024; unTreat-3w vs. Treat-3w, 25.8 ± 4.7% vs. 15.7 ± 9.8%, P = 0.314) (Figure 13).

Bladder overactivity due to I/R injury was confirmed to be the main cause of urothelial damage rather than degeneration of detrusor muscle layer.

4.2. Tempol treatment changed the expression of beta-3 receptors in the urothelium

To evaluate the mechanisms by which urothelial apoptosis causes functional changes, a Western blot experiment was conducted to identify changes in the expression of beta receptor protein in the urothelium.

The expression of beta receptors was increased in the experimental group compared to the sham group. The expression of the beta-3 adrenoreceptor was increased in the tempol-treated rats at three weeks after relief compared to the expression levels in the untreated rats. However, no significant difference in the expression of the beta-3 adrenoceptor was observed at one week after relief of pBOO between the treated and untreated rats (Figure 14).

Table 1. Changes in body and bladder weight

	Sham	Treat−1w	unTreat−1w	Treat−3w	unTreat−3w
Initial body weight (g)	205.2±15.5	204.1±11.2	209.2±9.2	200.0±14.4	209.9±11.8
Final body weight (g)	251.8±6.3	245.1±17.0	256.9±7.9	269.7±10.2	285.1±16.7
Bladder wet weight (mg)	12.5±7.6	26.9±9.7*	29.8±10.2*	20.5±7.1	25.3±8.8
Bladder wet weight/body weight (mg/g)	0.05±0.3	0.10±0.2*	0.11±0.4*	0.07±0.1	0.08±0.4

*P < 0.05 vs. sham (Mann−Whitney U test). Treat−1w, a rat group underwent a cystometrogram with treatment for one week after the reversal operation; unTreat−1w, a rat group underwent a cystometrogram without treatment for one week after the reversal operation; Treat−3w, a rat group underwent a cystometrogram with treatment for three weeks after the reversal operation; unTreat−3w, a rat group underwent a cystometrogram without treatment for three weeks after the reversal operation.

Table 2. Comparison of cystometric parameters between conscious sham-operated and obstructed rats

	Sham	pBOO	unTreat-1w	Treat-1w	unTreat-3w	Treat-3w
Basal pressure	18.6±3.4	22.6±8.9*	20.0±10.9	20.9±8.6	20.2±10.2	19.8±7.7
Threshold pressure	24.8±2.4	32.0±8.4*	39.3±13.3*	37.8±10.3*	34.1±20.9	25.7±12.6
Peak micturition pressure	58.8±15.5	103.6±27.8*	52.9±18.9	58.2±18.4	56.0±36.6	48.3±15.9
Micturition interval	378±115	244±91*	210±118*	205±56*	279±59	238±72*
Micturition duration	14.0±3.5	27.1±18.5	15.6±3.9	19.1±7.8	20.0±15.1	19.7±14.8
Voided volume	2.14±0.54	0.65±0.28*	1.31±0.79*	1.30±0.32*	1.81±0.35	1.52±0.46*
Non-voiding contraction	0	1.08±0.73*	1.18±0.82*	0.36±0.40*†	1.51±0.69*	0.23±0.25†
Bladder capacity	1.05±0.32	0.67±0.25*	1.40±0.79	1.37±0.37	1.86±0.39*	1.59±0.49*
Residual volume	0.15±0.21	0.02±0.13*	0.09±0.11	0.07±0.09	0.05±0.06	0.07±0.07

*$P < 0.05$ vs. the sham group; †$P < 0.05$ vs. the untreated group during the same period (Mann-Whitney U test). Pressure is expressed in mmH_2O; volume or capacity is expressed in mL; time is expressed in seconds. pBOO, partial bladder outlet obstruction; Treat-1w, a rat group underwent a cystometrogram with treatment for one week after the reversal operation; unTreat-1w, a rat group underwent a cystometrogram without treatment for one week after the reversal operation; Treat-3w, a rat group underwent a cystometrogram with treatment for three week after the reversal operation; unTreat-3w, a rat group underwent a cystometrogram without treatment for three week after the reversal operation.

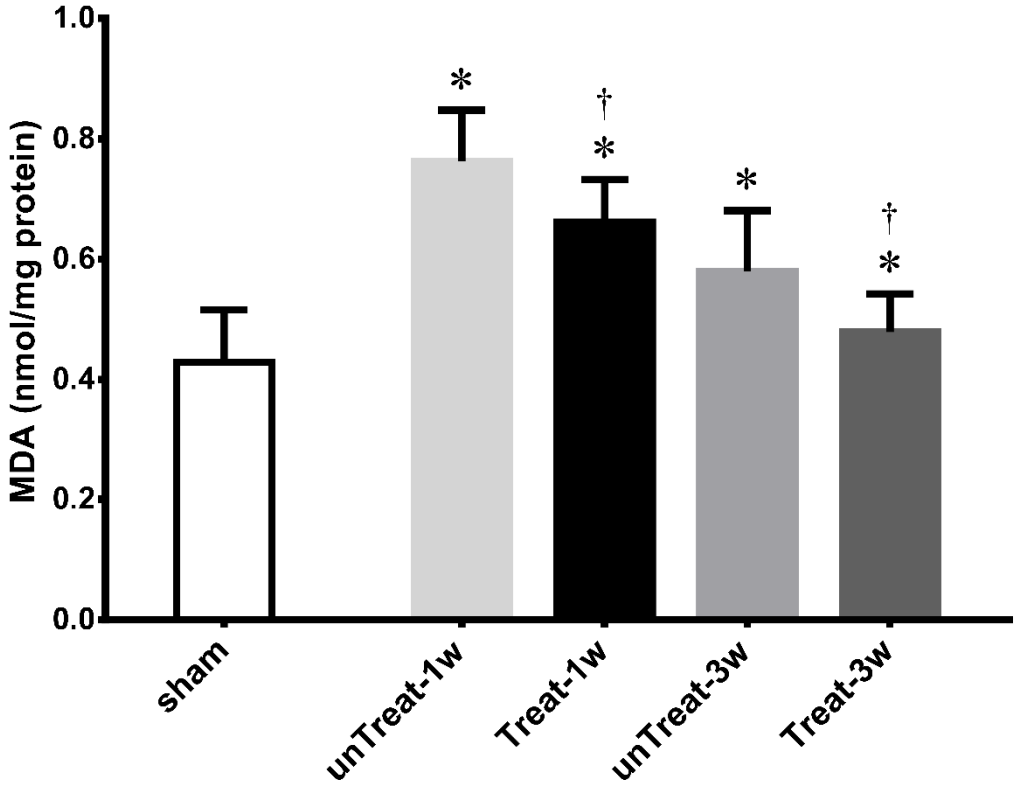


Figure 6. Malondialdehyde measurement in the bladder tissue. *P < 0.05 vs. the sham group; †P < 0.05 vs. the untreated group during the same period (Mann-Whitney U test). MDA, malondialdehyde; Treat-1w, a rat group underwent a cystometrogram with treatment for one week after the reversal operation; unTreat-1w, a rat group underwent a cystometrogram without treatment for one week after the reversal operation; Treat-3w, a rat group underwent a cystometrogram with treatment for three weeks after the reversal operation; unTreat-3w, a rat group underwent a cystometrogram without treatment for three weeks after the reversal operation.

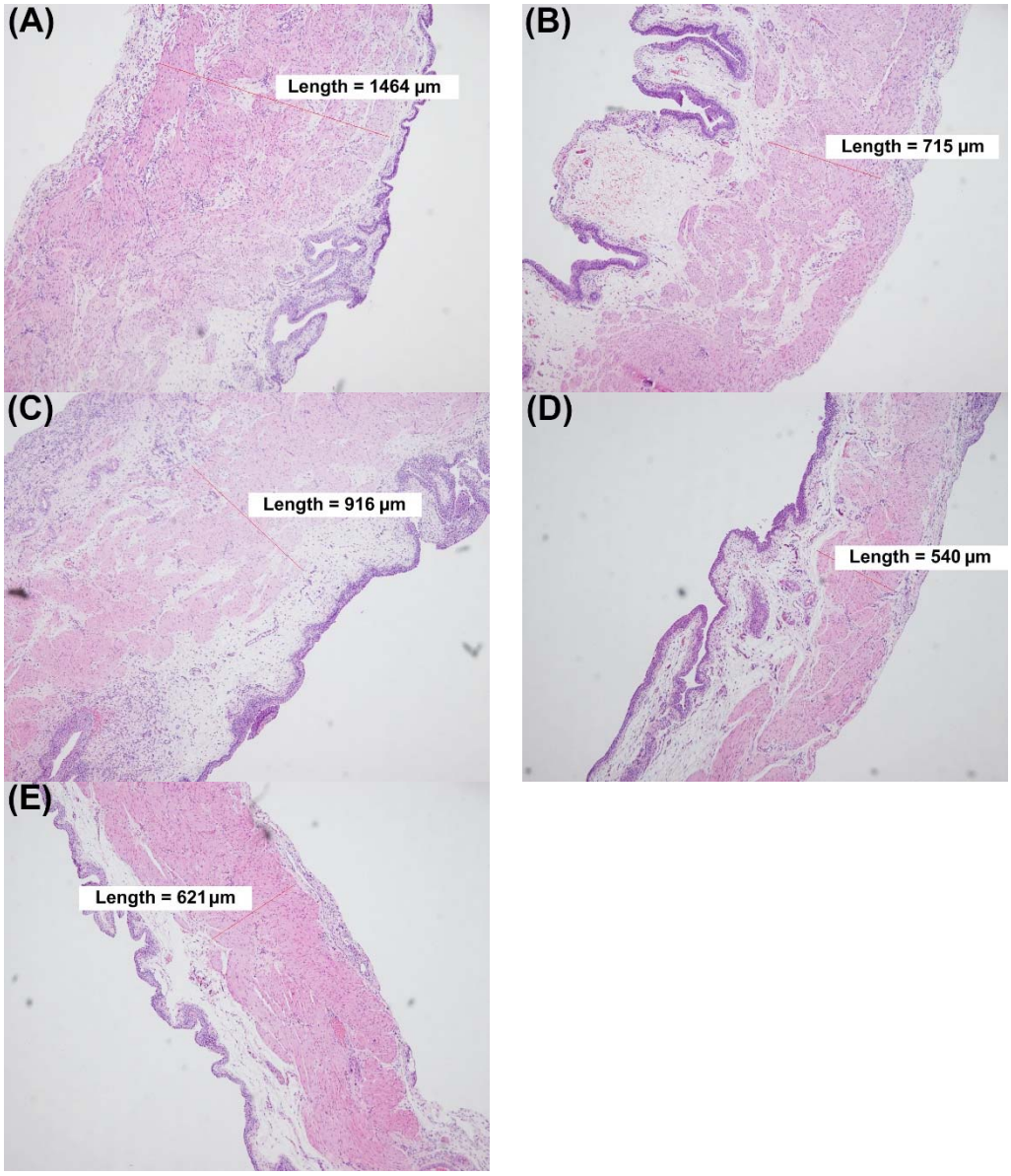


Figure 7. Changes in the detrusor muscle thickness after pBOO and its relief for one week and three weeks based on tempol treatment. Representative micrographs show a thin section of the bladder wall (magnification 100×). (A) untreated for one week; (B) tempol-treated for one week; (C) untreated for three weeks; (D) tempol-treated for three weeks; (E) sham.

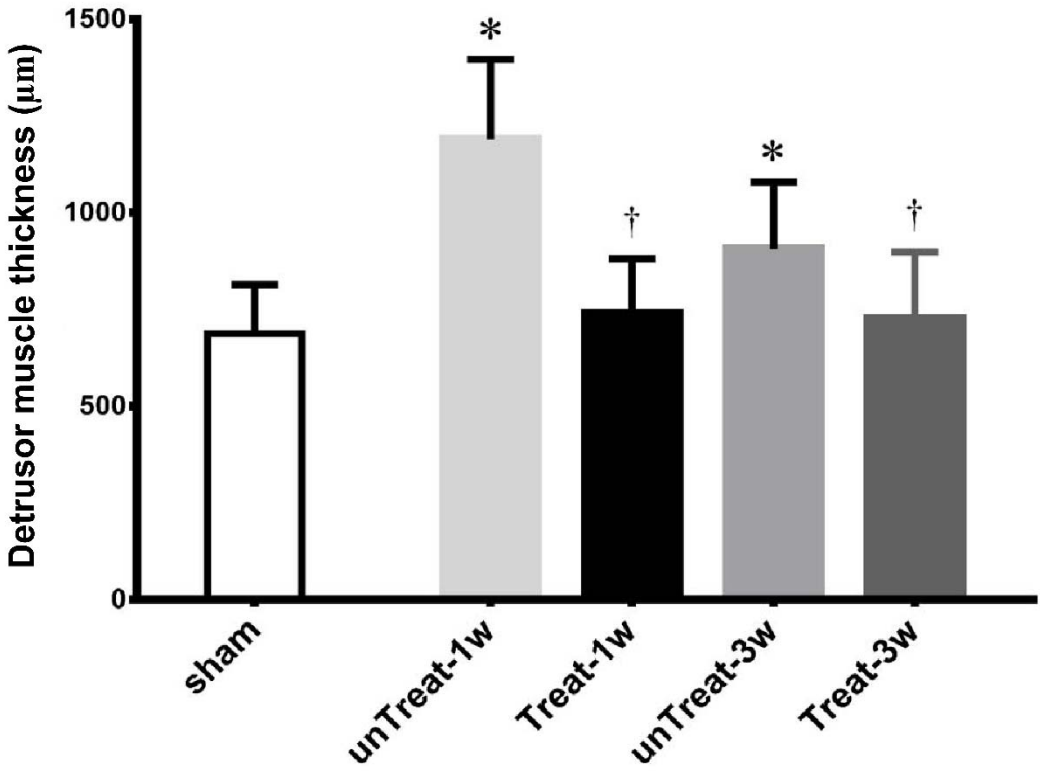


Figure 8. Changes in the detrusor muscle thickness after pBOO and its relief for one week and three weeks based on tempol treatment. The bar graph shows the quantitative image analysis. Results are expressed as the mean ± standard error of the mean. $^{*}P < 0.05$ vs. the sham group; $^{\dagger}P < 0.05$ vs. the untreated group during the same period (Mann-Whitney U test). Treat-1w, a rat group underwent a cystometrogram with treatment for one week after the reversal operation; unTreat-1w, a rat group underwent a cystometrogram without treatment for one week after the reversal operation; Treat-3w, a rat group underwent a cystometrogram with treatment for three weeks after the reversal operation; unTreat-3w, a rat group underwent a cystometrogram without treatment for three weeks after the reversal operation.

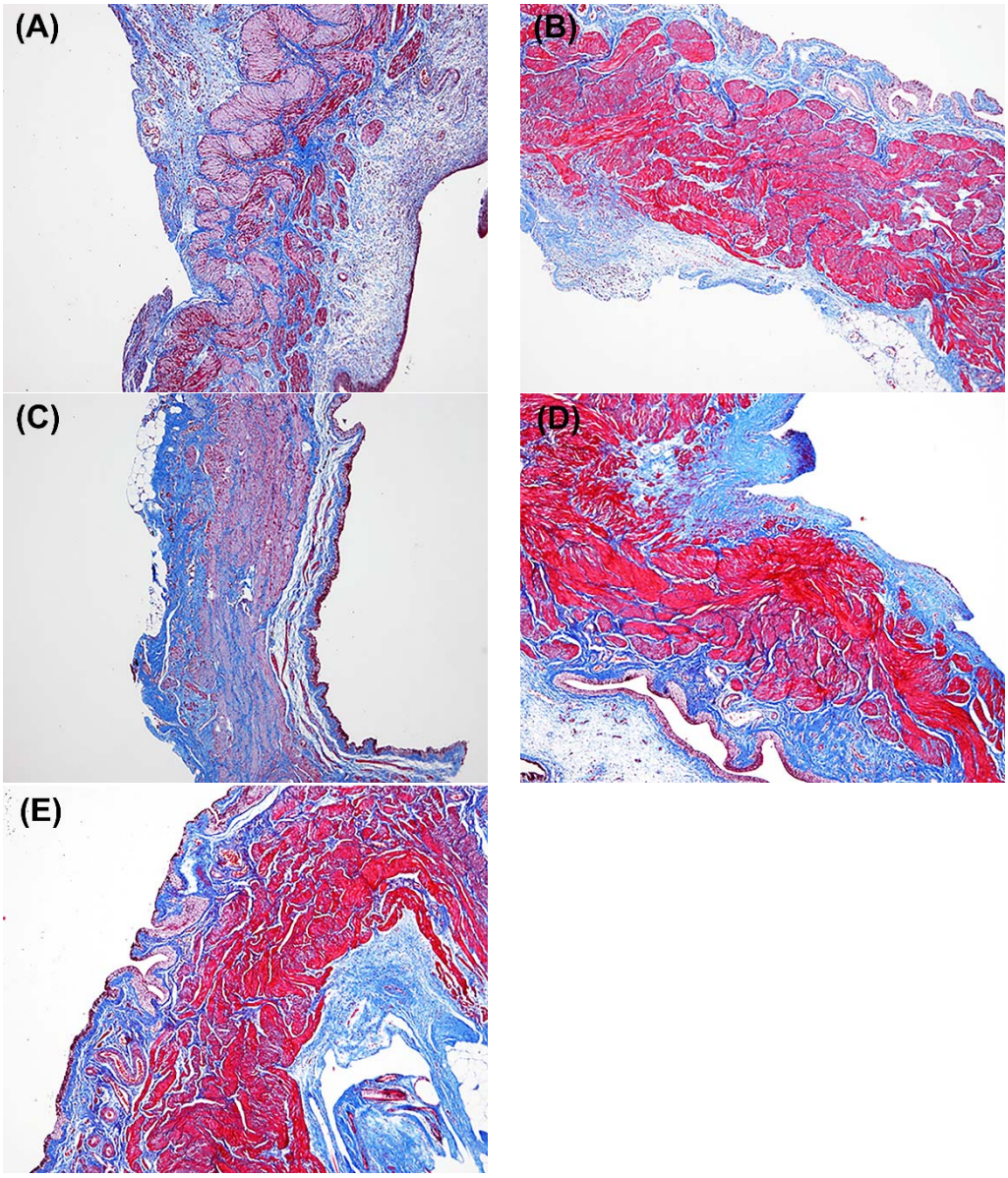


Figure 9. Masson's trichrome staining. Representative micrographs show collagen stained in blue and the muscle stained in purple (magnification 20×). (A) untreated for one week; (B) tempol-treated for one week; (C) untreated for three weeks; (D) tempol-treated for three weeks; (E) sham.

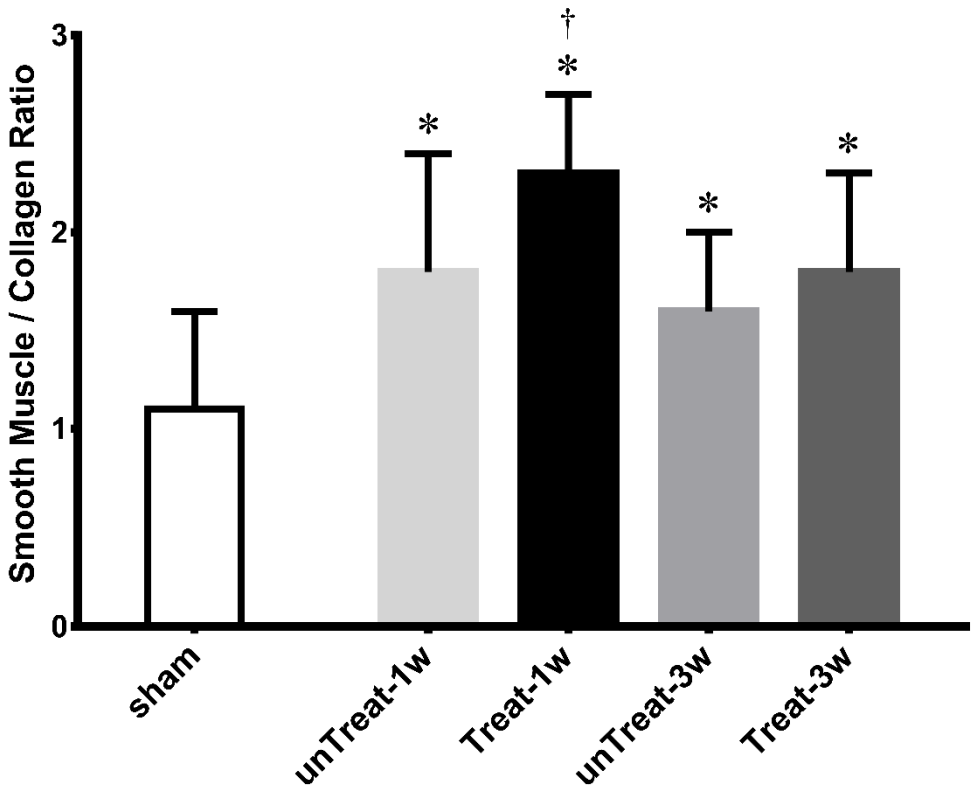
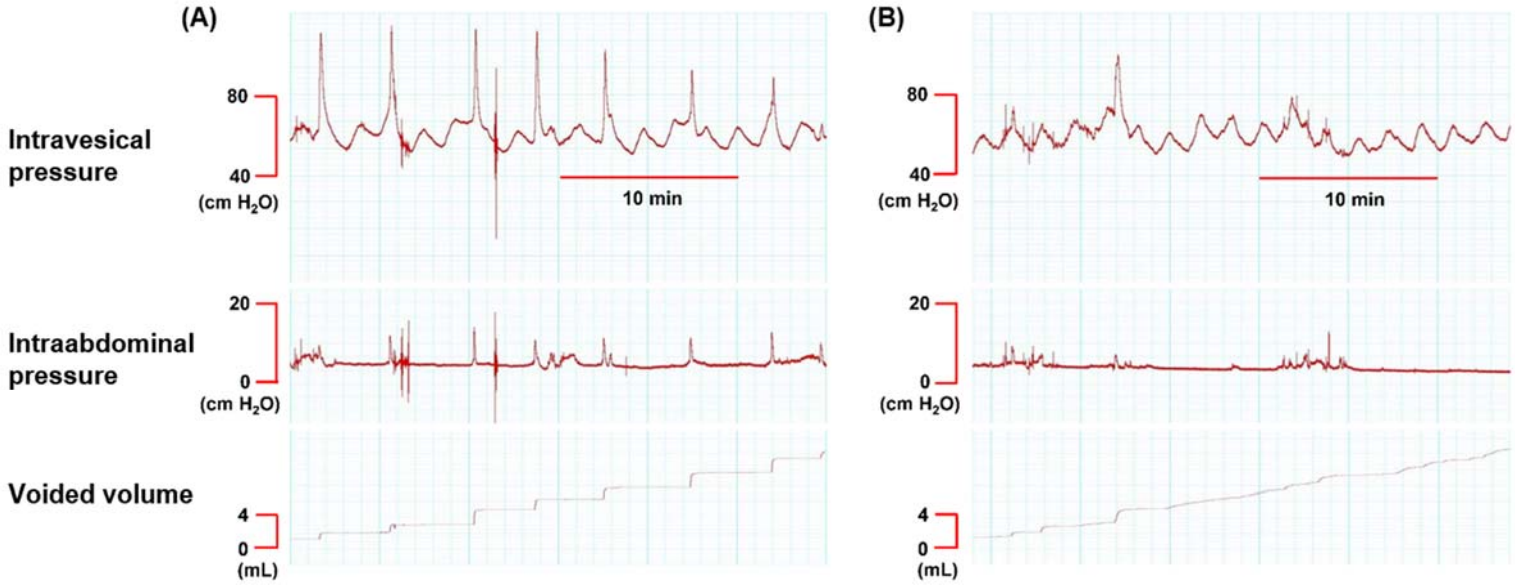


Figure 10. The ratio of collagen to smooth muscle in detrusor muscle layer. The bar graph shows the quantitative image analysis. Results are expressed as the mean ± standard error of the mean. *P < 0.05 vs. the sham group; †P < 0.05 vs. the untreated group in the same period (Mann-Whitney U test). Treat-1w, a rat group underwent a cystometrogram with treatment for one week after the reversal operation; unTreat-1w, a rat group underwent a cystometrogram without treatment for one week after the reversal operation; Treat-3w, a rat group underwent a cystometrogram with treatment for three weeks after the reversal operation; unTreat-3w, a rat group underwent a cystometrogram without treatment for three weeks after the reversal operation.


(A)
(B)
Intravesical pressure
Intraabdominal pressure
Voided volume
80
40
(cm H2O)
10 min
20
0
4
(mL)

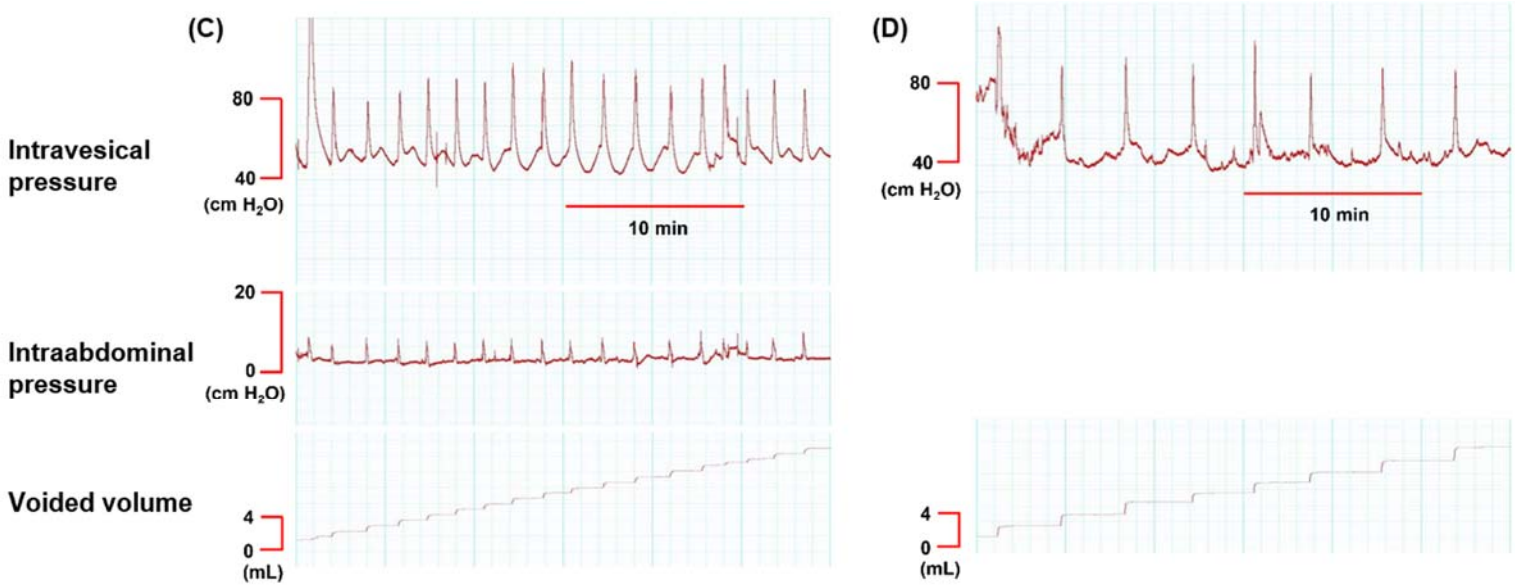


Figure 11. Representative cystometrographic recordings showing changes in intra-vesical pressure and intra-abdominal pressure at one week and three weeks after relief of partial bladder outlet obstruction according to tempol treatment. (A) untreated for one week; (B) untreated for three weeks; (C) tempol-treatment for one week; (D) tempol-treatment for three weeks.

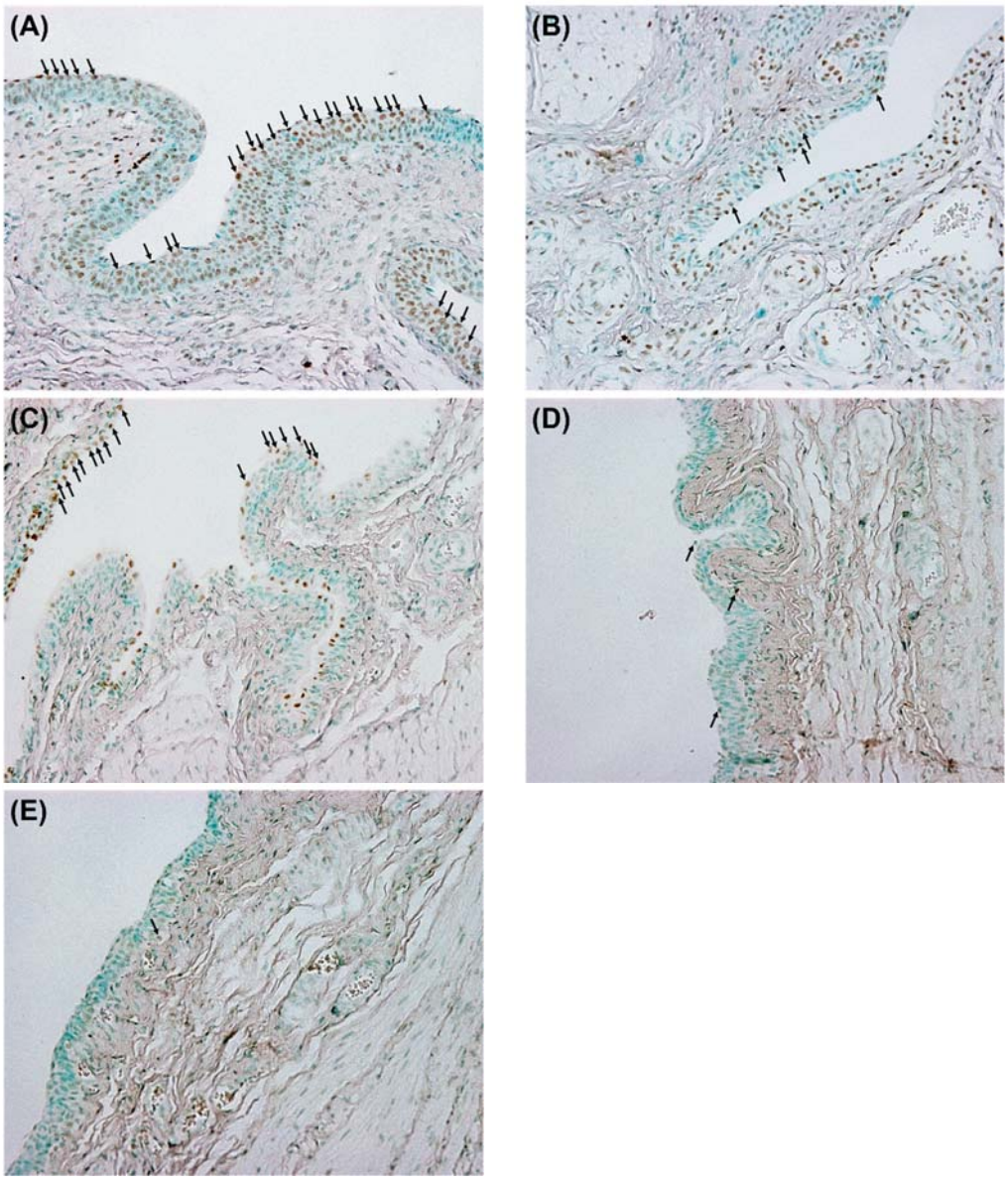


Figure 12. Detection of apoptosis. Representative micrographs show TUNEL-positive cells as black-brown cells mainly localized in the bladder urothelium (magnification 400×). Arrows indicate apoptotic cells. (A) untreated for one week; (B) tempol-treated for one week; (C) untreated for three weeks; (D) tempol-treated for three weeks; (E) sham.

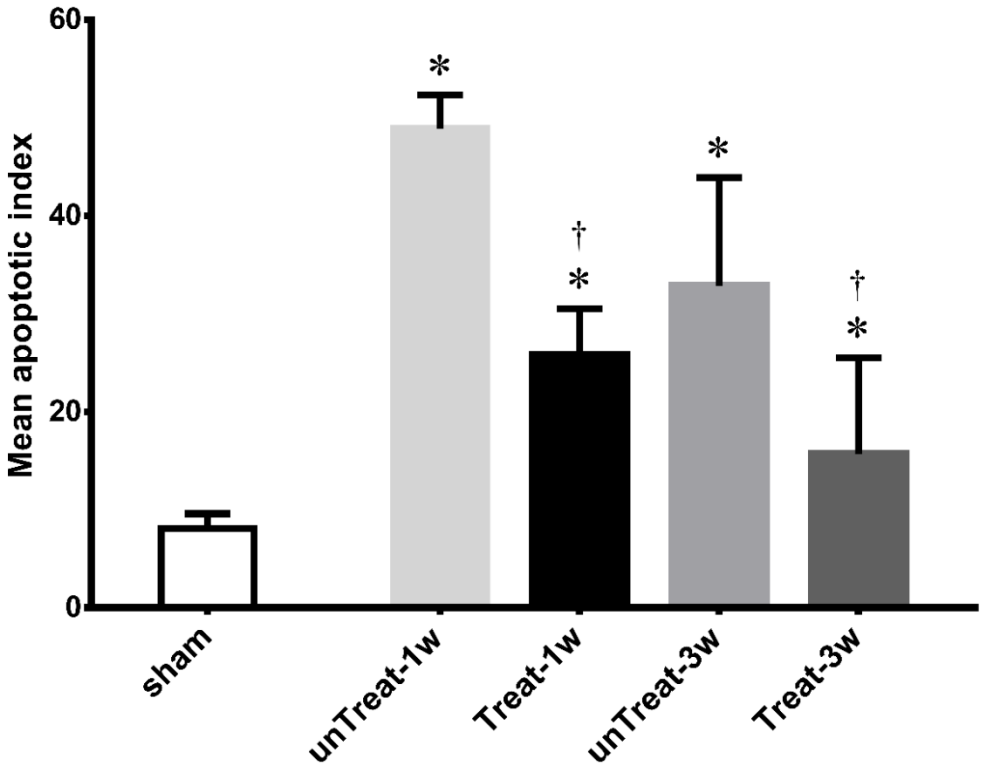


Figure 13. Detection of apoptosis. The bar graph shows the quantitative image analysis. The apoptotic index represents the percentage of apoptotic cells within the total number of cells in a given area. Results are expressed as the mean ± standard error of the mean. *P < 0.05 vs. the sham group; †P < 0.05 vs. the untreated group during the same period (Mann-Whitney U test). Treat-1w, a rat group underwent a cystometrogram with treatment for one week after the reversal operation; unTreat-1w, a rat group underwent a cystometrogram without treatment for one week after the reversal operation; Treat-3w, a rat group underwent a cystometrogram with treatment for three weeks after the reversal operation; unTreat-3w, a rat group underwent a cystometrogram without treatment for three weeks after the reversal operation.

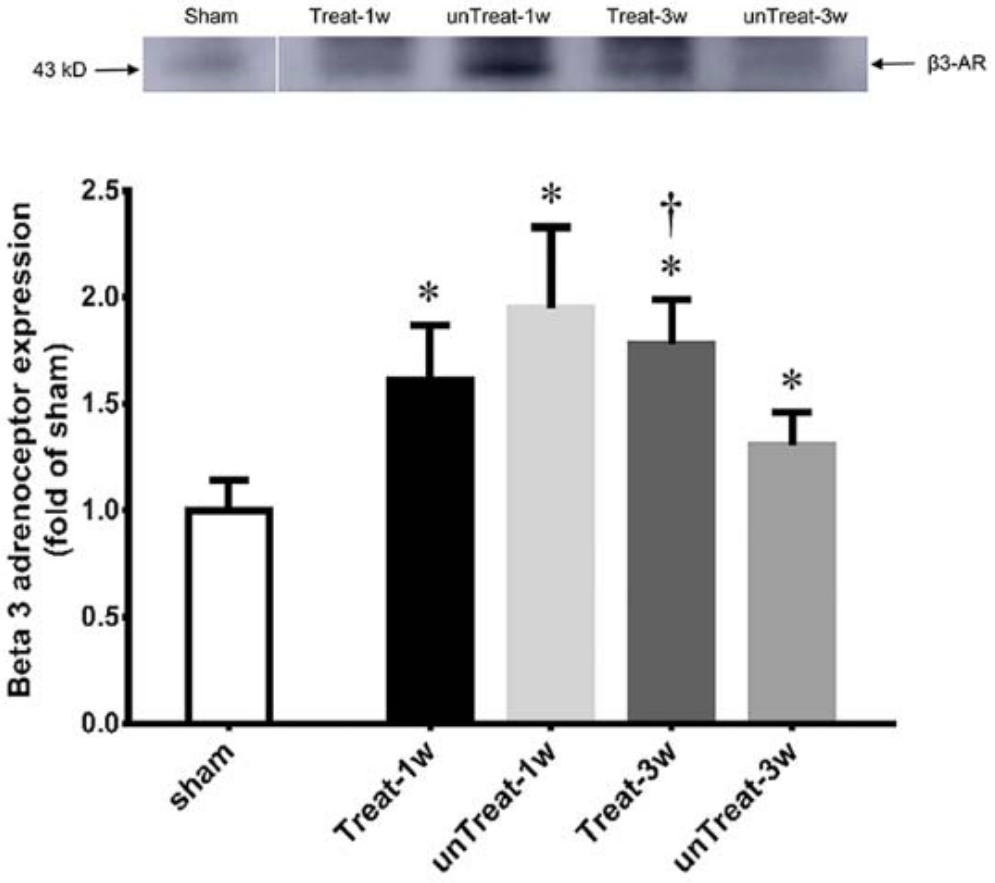


Figure 14. Western blot analysis. A representative Western blot shows expression of beta-3 receptor protein in the bladder. The bar graph shows the quantitative analysis of beta-3 receptor protein expression. Data are expressed relative to the corresponding expression in sham-operated rats. Results are expressed as the mean ± standard error of the mean. $^{*}P < 0.05$ vs. the sham group; $^{\dagger}P < 0.05$ vs. the untreated group during the same period (Mann-Whitney U test). Treat-1w, a rat group underwent a cystometrogram with treatment for one week after the reversal operation; unTreat-1w, a rat group underwent a

cystometrogram without treatment for one week after the reversal operation; Treat-3w, a rat group underwent a cystometrogram with treatment for three weeks after the reversal operation; unTreat-3w, a rat group underwent a cystometrogram without treatment for three weeks after the reversal operation.

Discussion

This animal study has shown antioxidant agents, such as tempol, help prevent bladder I/R injury after relief of pBOO. The preventive effects were demonstrated at one and three weeks. This preventive effect of antioxidants on I/R injury was shown to reduce apoptosis mainly in the mucosal layer and to restore muscle hypertrophy more rapidly in the smooth muscle layer. This effect resulted in a decrease in NVCs in cystometrogram that was presumably related to beta-3 adrenoreceptor expression.

pBOO causes a chronic ischemic status in the bladder. High pressure due to sustained overdistention can induce a reduction of blood flow to the bladder wall (Chai et al. 1999). Additionally, pBOO increases the thickness of the bladder wall through hypertrophy of the detrusor smooth muscle and deposition of collagen tissues, resulting in a reduction of microvascular blood perfusion (Tong-Long et al. 1998). Furthermore, rapid reperfusion due to relief of pBOO in chronically ischemic bladders leads to the generation of free radicals and rapid oxidative stress. Relief of pBOO resolves the intravesical high pressure, although resolving the bladder wall thickness takes at least three weeks, and the ongoing reduction of microvascular blood perfusion lasts for a

considerable period. The increased metabolic demand on the hypertrophied detrusor can produce more severe damage in the bladder when combined with a reduction in microvascular blood perfusion (Levin et al. 1999). The decompensated bladder also causes substantial residual urine, and overdistension of the bladder persists for a substantial period of time, resulting in sustained I/R injury after relief. In the present study, this I/R injury persisted for up to three weeks after relief of pBOO.

Chronic I/R injury induced NVCs in the cystometrogram in this study. Oxidative stress results in the generation of free radicals and oxidative damage (Bisogni et al. 2012). Chronic oxidative stress may damage intrinsic nerves, resulting in partial denervation of smooth muscle. Denervation of the detrusor muscle leads to sensitization of the afferent pathway through postjunctional supersensitivity with increased neurotransmitters and upregulation of neurokinin receptors (Nomiya et al. 2015). Denervation and nerve degeneration in sensory pathways during chronic I/R injury also cause an increase in the nerve growth factor level that may induce bladder hypersensitivity (Azadzoi et al. 2007). Additionally, chronic oxidative stress leads to the accumulation of calcium in the intracellular medium and the formation of metabolic end products that damage the detrusor musculature (Bisogni et al. 2012).

The resulting neurogenic and myogenic damage may cause detrusor overactivity but impaired contractility, resulting in NVCs in the cystometrogram.

Oral administration of antioxidants reduced I/R injury of the bladder after relief of pBOO. Several studies have proven that antioxidants reduce oxidative stress. Antioxidants have shown preventive and therapeutic benefits in several cardiovascular diseases (Jain et al. 2015). Free radical scavengers decrease blood pressure and improve vascular function via biochemical mechanisms, such as normalizing the increased renal sympathetic nerve activity, plasma norepinephrine levels, and angiotensin type I receptor expression and enhancing carotid body chemoreceptor sensitivity to hypoxia (Rosenbaugh et al. 2013). The use of antioxidants after liver transplantation attenuates the effects of I/R–related oxidative stress and reduces lipid peroxidation (Barros et al. 2015). However, few reports have shown that antioxidants can reduce oxidative stress in the bladder. I/R injury after BPH surgery has drawn increasing attention with the popularity of the holmium laser enucleation of the prostate (HoLEP) procedure, which can completely resect the adenoma. Determining whether the delivery of stable antioxidants to the target organ is possible is important at the time of systemic administration of antioxidants. The results of the present study

demonstrated that effective delivery of antioxidants to the bladder was also possible. However, studies on bladder-specific delivery may be necessary in the near future.

Apoptosis due to I/R injury was mainly observed in the mucosal layer rather than in the muscle layer. Sensory neurons in the lamina propria, which are susceptible to I/R injury, may be irreversibly damaged by I/R injury (Steers et al. 2002). Degeneration of sensory neurons in the mucosal layer may contribute to detrusor instability. In this study, more NVCs occurred in the untreated rats, which were not given antioxidants and sustained more I/R injuries. This finding could be inferred from the influence of apoptosis in the mucosal layer. This finding of present study suggested that the pathophysiology of an overactive bladder may be due to the neuroplasticity of peripheral sensory neurons rather than the myogenic hypothesis.

A difference in beta-3 adrenoceptor expression was observed depending on the degree of I/R injury. In this study, the administration of antioxidants for three weeks prevented reduction of beta-3 adrenoceptor protein expression compared to the expression level in the untreated group. Recent studies have found that the expression of beta-

3 adrenoceptor mRNA may be dependent on the degree of obstruction. However, the results are still controversial. In the severe BOO group, the expression level of beta-3 adrenoceptor mRNA in the mucosa of the prostatic urethra was significantly lower than that in the mild BOO group (Kurizaki et al. 2013). In contrast, pBOO has been reported to increase beta-3 adrenoceptor mRNA expression in the bladder of rat models (Park et al. 2010). Activation of beta-3 adrenoceptor in the urothelium induces relaxation of the bladder detrusor muscle (Yamaguchi et al. 2007). Clinically, some patients exhibit no effect of beta-3 agonists. The difference in the effect of beta-3 agonists may be explained by the differences in beta-3 adrenoceptor expression in the urothelium of the bladder.

After surgery for BPH, such as HoLEP, newly developed bladder overactivity, which was not observed before surgery, has been reported in as many as 40% of patients (Cho et al. 2014). There is no adequate treatment for patients with de novo urgency urinary incontinence after HoLEP surgery. Commonly used agents in patients with overactive bladder, such as antimuscarinics or beta-3 agonists, are not effective in this situation. The findings of present study suggest that changes in beta-3 receptor expression following I/R injury may result beta-3 agonists being ineffective in patients with de novo urgency urinary

incontinence. Therefore, in patients with severe trabeculation or with a functionally decompensated state, it may be clinically meaningful to prevent additional I/R injury with free radical scavengers after HoLEP surgery. Prior to clinical application, antioxidants that can be effectively delivered to the bladder should be developed and screened.

Conclusions

Treatment with an oxygen free radical scavenger demonstrated preventive effect on morphological and functional changes in the urinary bladder at one and three weeks after relief of pBOO. Morphologically, tempol treatment prevented detrusor hypertrophy and deposition of collagen fibers in the detrusor muscle layer. Functionally, tempol treatment prevented detrusor overactivity. These preventive effects were associated with a decrease in apoptosis in the urothelium and a change in beta-3 receptor expression. Further studies are needed to clarify the biochemical mechanisms of the changes in beta-3 receptor expression and the effect of these changes on bladder function.

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초록

흰쥐 만성 부분 방광출구폐색 (partial bladder outlet obstruction, pBOO) 모델을 이용하여 폐색 해소 후 산소유리기제거제의 투여가 방광의 형태학적 및 기능적 변화에 미치는 효과를 알아보고자 하였다.

8주령 암컷 Sprague-Dawley 흰쥐 (200-250g) 40마리의 pBOO 모델을 만들고, 3주후 폐색 해소를 하였다. 폐색 해소 후 산소유리기제거제인 tempol로 1주 혹은 3주간의 치료군과 비치료군 각 10마리씩, 총 4군의 실험군과 Sham 수술 대조군을 비교 분석하였다. Awake cystometrogram (CMG)으로 방광의 기능적 변화를 평가하였다. CMG 직후 방광을 적출하여 무게를 측정하였다. 적출된 방광 조직에서 malondialdehyde (MDA)를 측정하여 산화 스트레스 정도를 확인하였다. 또한 hematoxylin and eosin (H&E) 염색과 Masson's trichrome 염색으로 형태학적 변화를 관찰하였고, TUNEL 염색과 Western blot 실험으로 방광 변화의 기전을 파악하고자 하였다.

MDA로 측정한 폐색 해소 후 방광의 산화스트레스는 tempol 치료군에서 의미 있게 감소함을 치료 1주와 3주에서 모두 확인하였다. pBOO 유발 실험군의 방광 무게는 sham 군에 비해 증가하였으나, 치료 유무와 치료 기간에 따른 군간의 유의한 차이는 없었다. H&E 염색에서는 pBOO 유발 후 배뇨근의 비후가 명확하게 관찰되었고, 폐색 해소 3주 후에는 배뇨근 두께가 의미 있게 감소하였다. Tempol 1주 치료군과 3주 치료군 모두에서 배뇨근층의 두께가 비치료군에 비해 유의하게 감소하였다. 배뇨근층 사이의

콜라겐 섬유의 침착도 tempol 치료군에서 비치료군에 비해 의미 있게 감소함을 확인하였다. Awake CMG를 통해 확인한 방광의 기능적 변화는 Tempol 치료군에서 비치료군에 비해 배뇨주기당 non-voiding contraction의 발생이 유의하게 감소하였다 (1주 비치료군 vs. 치료군, 1.2 ± 0.8 vs. 0.4 ± 0.4, P = 0.010; 3주 비치료군 vs. 치료군, 1.5 ± 0.7 vs. 0.2 ± 0.3, P = 0.002). TUNEL 염색으로 관찰한 세포고사는 요로상피층에서 주로 관찰되었고, 1주와 3주 모두에서 고사된 요로상피세포의 비율이 치료군에서 유의하게 감소하였다. Western blotting으로 확인한 베타-3 수용체의 발현은 pBOO를 유발한 실험군에서 sham군에 비해 증가함을 관찰하였다. 폐색 해소 1주 후에는 베타3 수용체 발현이 치료군과 비치료군에서 차이가 없었으나, 폐색해소 3주 후에는 치료군에서 베타3 수용체 발현이 유의하게 증가되어 있음이 확인되었다.

산소유리기제거제는 부분 방광출구폐색 해소 과정에서 발생하는 산화스트레스에 의한 방광의 변화들을 예방하였다. 형태학적으로 배뇨근의 비후와 배뇨근층 사이의 콜라겐 섬유의 침착을 감소시켰고, 기능적으로 방광과민성을 예방하였다. 이 과정에 방광 요로상피층의 세포고사와 베타-3 수용체의 발현이 관여하는 것을 확인하였다.

*이 연구의 상당 부분은 PLoS One 저널에 게재되었음 (2018년 13권 10호 e0199800).

주요어: 방광출구폐색, 과민성방광, 산화스트레스, 산소유리기제거제, 베타3 수용체

학번: 2013-30575